Variation in Time of Egg Hatch by the Honey Bee, *Apis mellifera* (Hymenoptera: Apidae)

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ABSTRACT More detailed information on the age at which a honey bee, *Apis mellifera* L., egg hatches and the natural variation of this trait was needed to guide development of cryopreservation technology for honey bee embryos. Therefore, honey bee queens were caged on a clean, empty comb for 4 h to obtain groups of eggs of known age. These eggs were collected from the comb using a special forceps and placed on beeswax-coated petri dishes. Individual eggs were observed from 65 h after oviposition until they hatched (48.6% hatched). A tracheal network became visible ≈2 h before hatching. Then, slow flexing of upright embryos and abdominal peristalsis were seen. Release of a fluid along the dorsal midline of the embryo was observed rarely in normal hatching. In contrast, fluid was frequently observed seeping from bulges on embryos that hatched poorly (30.6%). In a normal sequence, the eggshell was gradually digested away, and complete hatch accomplished. The age at which this occurred was significantly different between eggs from different queens, ranging from 66 to 93 h. Hatching age may be a useful marker for selection of faster development time overall, a possible mode of resistance to the varroa mite. Respiration was visible in the larvae for 1–9 h after hatch. In vitro rearing procedures for embryos preserved by cryopreservation will be designed around the parameters estimated in this study.

KEY WORDS embryo, eggshell, hatch, genetic variation, honey bee

HONEY BEE, *Apis mellifera* L., eggs hatch in an unusual manner. Most insect larvae hatch by mechanical disruption of the eggshell (chorion plus vitelline membrane), which has a region of structural weakness to facilitate breaking (Magaratis 1985). In contrast, the honey bee secretes a solution that digests the eggshell completely (DuPraw 1961). Some other Hymenoptera, such as the carpenter bee, *Xylocopa virginica* L., also dissolve the membranes (Fyg 1962). DuPraw (1961) gave the first complete verbal description of the stages involved: flexing of the embryo in a dorsal-ventral plane, appearance of a droplet of liquid that disperses across the egg surface, increasing visibility of the segmentation as the chorion "collapses," eventual repositioning of the larva onto it's side, and initiation of respiration. He also speculated that the digestive solution that releases the embryo was sequestered between the chorion and the vitelline membrane until the movements of the larva created a break allowing the liquid to spread onto the eggshell surface where it could be active. The presence of any fluid, even royal jelly, in the comb cell will prevent hatching, probably by diluting or interfering with the action of the digestive material.

The time of hatching of the honey bee egg is generally reported in beekeeping literature to be 3 d (72 h) of age (Laidlaw 1979) based on observations by Bertholf (1925), but there is variation. Nelson (1915) reported 74–76 h from his work on honey bee embryology. DuPraw (1961) reported the general range to be 72–76 h from his extensive observations of hatching during further embryological studies. Harbo et al. (1981) compared the development times of European and Africanized honey bee queens in Venezuela and found means of 73.3 ± 1.14 and 69.9 ± 1.06 h, respectively. Milne et al. (1988), who were observing embryo development under oil, found complete hatching to occur at 76.7 ± 2.1 h.

I am currently working on the development of cryopreservation technology for the honey bee embryo, based on protocols from *Drosophila melanogaster* L. (Steponkus et al. 1990, Mazur et al. 1992). Each step in the process of preparation of the embryo for freezing must be adapted to the biology of the honey bee. Success at each step is evaluated by the number of treated embryos that can successfully develop and hatch. To make the observation of newly hatched larvae more efficient, without missing those that might hatch earlier or later than the expected 72 h, I wanted to have more detailed information on the timing of the process and whether there were visible changes to signal that hatching was imminent.

The ultimate measure of success of embryo cryopreservation will depend on the number of living lar-

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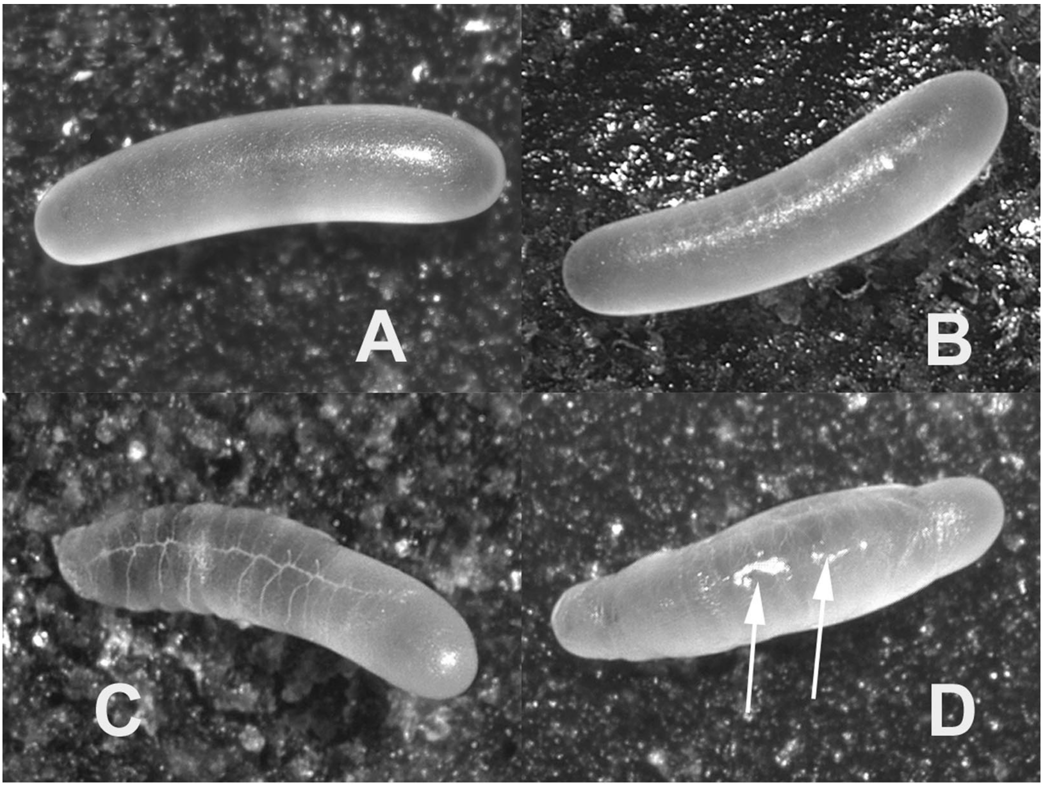

Fig. 1. First three stages of honey bee egg hatching. (A) An egg, as it appears from oviposition until a few hours before hatching begins. (B and C) Early and late stage appearance of tracheal network and segmentation. (D) Release of fluid to digest eggshell, along dorsal midline (arrows).

vae that can be thawed from the frozen state and reared as queens. In a colony, newly hatched larvae are fed with mandibular secretions by nurse worker bees. Knowing the range of hatching times and how long larvae can survive without being fed will be critical to an in vitro rearing system. Cryopreserved larvae will need to be fed until they can be moved into a queen-rearing colony. Normal commercial queen production involves transferring larvae less than 2 d old from their home colony into a queenless colony where they will be fed copious amounts of royal jelly to develop into queens. Therefore, this study was done to provide information on the timing of the hatching process under the incubation conditions used for the cryopreservation research, to identify visual cues of hatching progress, and to determine the durability of newly hatched larvae.

Materials and Methods

Fifteen mated, laying queens from two different commercial breeders were established in two-story, five-frame nucleus colonies. The queens were caged under wire mesh cages (10 cm^2) on a clean, empty comb and returned to their own colony for 4 h. Previous experience with egg laying under these cages showed that queens rarely laid any eggs within the first 2 h, and an interval of 4 h was necessary to achieve sufficient numbers of eggs for an experiment. Accordingly, I used hour 3 of each 4-h caging period ± 1 h as the estimated mean time of laying for the group of eggs in each cage. All laying occurred between 1145–1435 h. At the end of the caging period, the queens were released. The newly laid eggs were recovered with the cage, and the frame replaced in the center of the brood nest for 2 d.

The frames with cohorts of aged eggs (21–23 h) were brought into the laboratory and held in a 35°C incubator until the eggs could be collected with a Taber forceps (Taber 1961, Collins 2002) that was designed to cradle, not crush, the egg. A queen normally lays a single egg in each cell of the wax comb, with the posterior end of the cylindrical egg glued to the bottom of the cell. The middle of the egg was clasped with the forceps, and the egg plucked free and transferred in the same upright position to beeswax surfaces on the bottom of 5-cm glass petri dishes. Each dish had eggs from only one queen. To make the eggs more visible, the beeswax was colored by the addition of crayon wax when it was melted and poured into the petri dishes. The waxed surfaces were marked into grids such that individual eggs could be identified and followed over time. The covered petri dishes were held in an incubator at 35 ± 1°C and >95% RH.

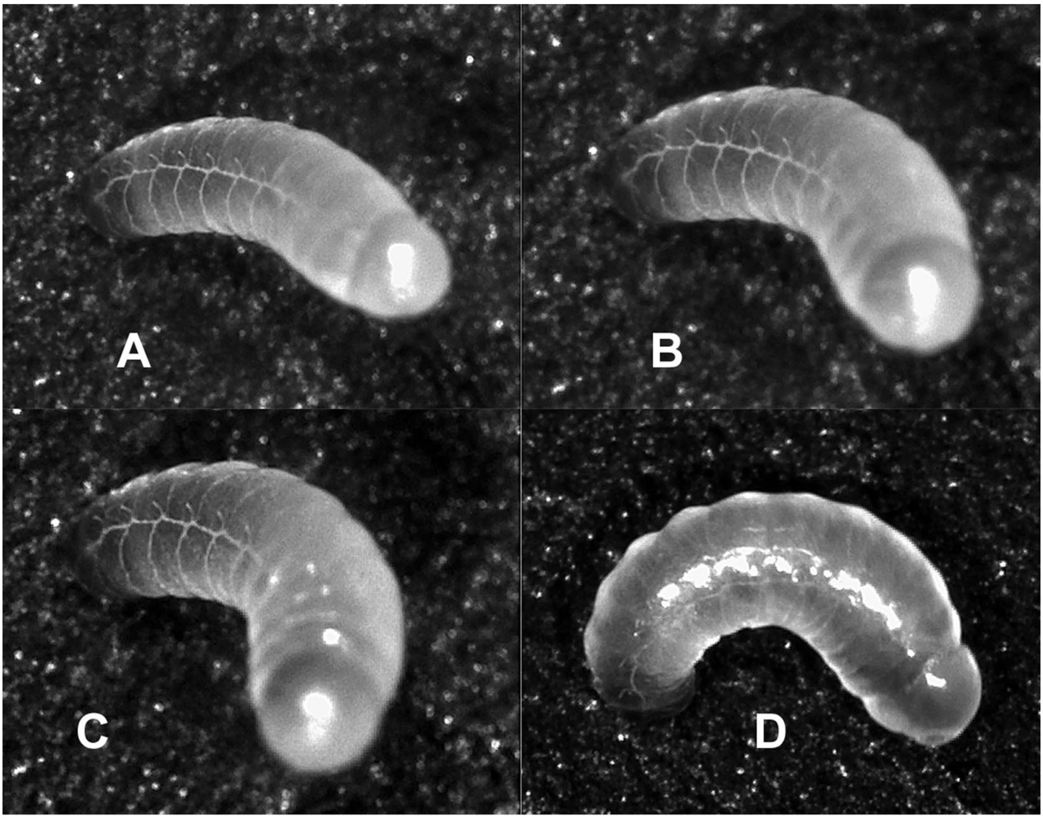

Fig. 2. Dorsal-ventral flexing of embryo from upright position to prone, where it assumes a C shape. The line of the tracheal network is along each side. Flexing occurs before, during, and/or after eggshell digestion.

At irregular intervals determined by the progression of hatching, the eggs were removed from the incubator and observed under a dissection microscope (SV11; Carl Zeiss GmbH, Jena, Germany). The mean interval between observations was 102 ± 30 min, and observations were made around the clock until no more eggs hatched. Once hatching began, observation intervals were shortened (41 ± 19 min). The first replicate lasted only 18 h but missed some of the early and late hatching, so the next five replicates lasted for 26–30 h. The time of hatch was noted when a larva was observed to be completely free of the eggshell. Time out of the incubator for collection and observation was <1 h total over all observation periods. Photomicrographs were taken of the visibly different stages of hatching using a Zeiss Axiocam digital camera (Carl Zeiss, Thornwood, NY). The mean time of completed hatch was compared between colonies by an analysis of variance (ANOVA, Proc GLM; SAS Institute 2000).

Results and Discussion

Nine of the 15 queens laid a sufficient number of eggs (>20) during at least one of the caging periods and were included in the observations. No queen laid eggs every time, but two were frequent layers and were included in four of the six testing periods. Of the 1,144 eggs collected, 550 (48.1%) hatched successfully, 179 (15.6%) were damaged, 84 (7.3%) remained unchanged (stayed as eggs) during the entire observation period, and 217 (19.0%) began an abnormal sequence (described below) and instead of hatching dried up or decomposed along with the remaining eggs (114; 10.0%). The damaged group included 104 eggs that dried out rapidly after rupture of the membranes, 2 that were bent, 14 that had a blotchy appearance indicating abnormal development (DuPraw 1960, Milne et al. 1988), 23 that appeared to begin to hatch but never completed the process, and 36 that were no longer present or visible in the dish when observations began. Harbo (1981) hatched eggs in comb in an incubator and reported that one-half of naturally mated queens had <5% egg mortality, and 80% had <10% egg mortality. Egg damage during transfer out of the comb probably accounts for much of the current mortality.

Most of the stages observed during this study were similar to those described by DuPraw (1961), Milne et al. (1988), and Nelson (1915). During normal development, the egg maintains its shape and opaque white color until just before hatching (Fig. 1A). The onset of hatching is signaled by a growing transparency of the embryo and the appearance of a white tracheal network (Fig. 1, B and C). This occurs 167 ± 109 min

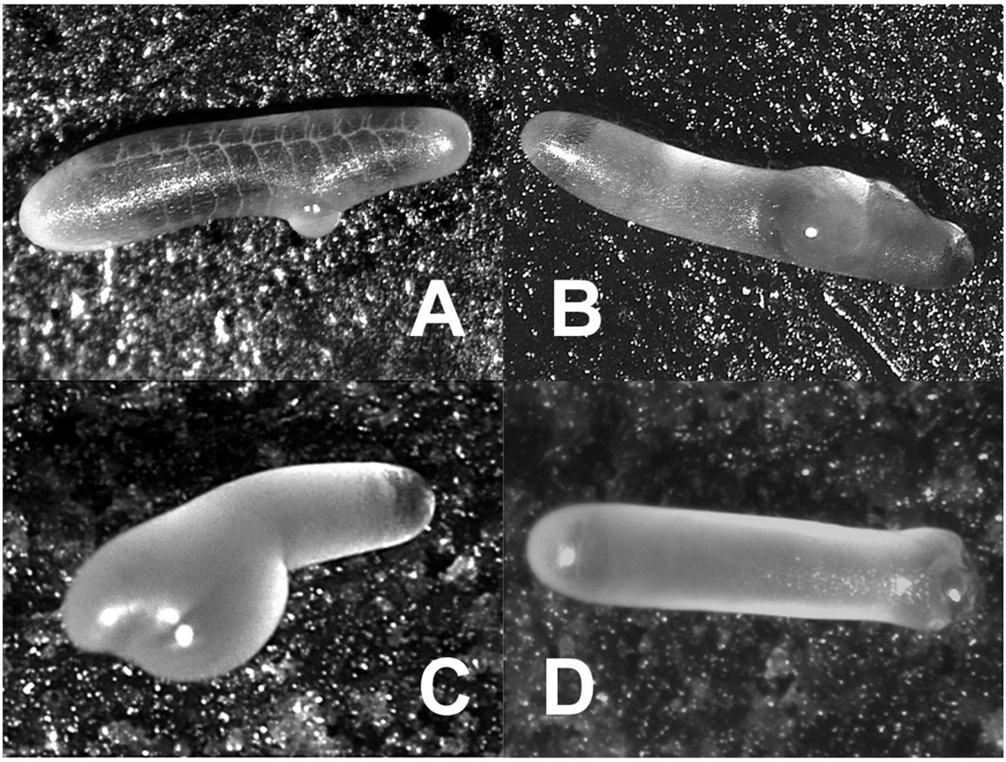

Fig. 3. Four examples of abnormal bulging and fluid release. These eggs have a reduced (30.6%) probability of hatching. (A) A large droplet on dorsal midline. Bulge and oozing liquid near egg base (B) or top (C). (D) A droplet at the posterior end (base, right).

(N = 60) before complete hatching. The visibility of the tracheal system at this time may be caused by the secretion of gas into the tracheae, as observed with other species of insects (Sander and Gutzeit 1985). The appearance of the tracheal net was not mentioned by previous authors describing the honey bee.

About 1 h later (115 ± 56 min before hatch, N = 13), the upright embryo may begin slow rocking motions, dorso-ventrally (Fig. 2, A–C). This flexing continues with increased frequency until the embryo bends itself over with its head touching the surface (Fig. 2D). This serves to move the mouthparts into position near the puddle of brood food that will be deposited on the base of the cell. From there, it will slowly fall onto its side and assume the normal C shape of the larval stage. If the egg is already lying down on the beeswax, as happens to some during transfer, this flexing does not occur, but the larva assumes the same C shape.

DuPraw (1961) suggests that this weaving action also breaches the eggshell to release the digestion fluid. Figure 1D shows a prone embryo with a small amount of liquid (light reflection) escaping along the midline of the dorsal surface. This was only rarely seen with normal eggs; therefore, the process probably takes place rapidly. For one round of observations, the eggs were scored by whether they were upright (80) or prone (86). These hatched in equal numbers, 68 and 67, respectively as did eggs observed by Dietz (1964). Thus, flexing does not serve as the method to release the solution that dissolves the eggshell.

Figure 3 shows variations in the release of the digestion liquid among embryos. What appears to be a large droplet of liquid on the surface is only a thin layer. When the liquid was removed with a small piece of tissue, it was seen that the embryo itself had bulged at this location. This bulge may be present for a long time (maximum 312 min), and only 30.6% (110 of 327) of the eggs showing this feature successfully hatched. However, for those abnormal eggs that did hatch, the times fell into the same range as for the normal eggs. The bulge may appear at any location on the embryo. This bulging, which seems to be associated with abnormal secretion of digestive fluid, may signal a developmental problem, probably also caused by damage during transfer. The question remains whether this larva can be reared into a functional queen or is poorly developed in some way.

Like other insects (Magaratis 1985), escape from the eggshell by the honey bee occurs by peristaltic movements of the abdomen (Fig. 4A). Waves of contraction were observed as early as 134 min before hatching was complete (48 ± 34 min, N = 13) and as long as 63 min after the larva was completely free of eggshell (38 ± 22 min, N = 3). In some instances, the early peristalsis was seen before any evidence of eggshell degradation. Contractions can persist over long

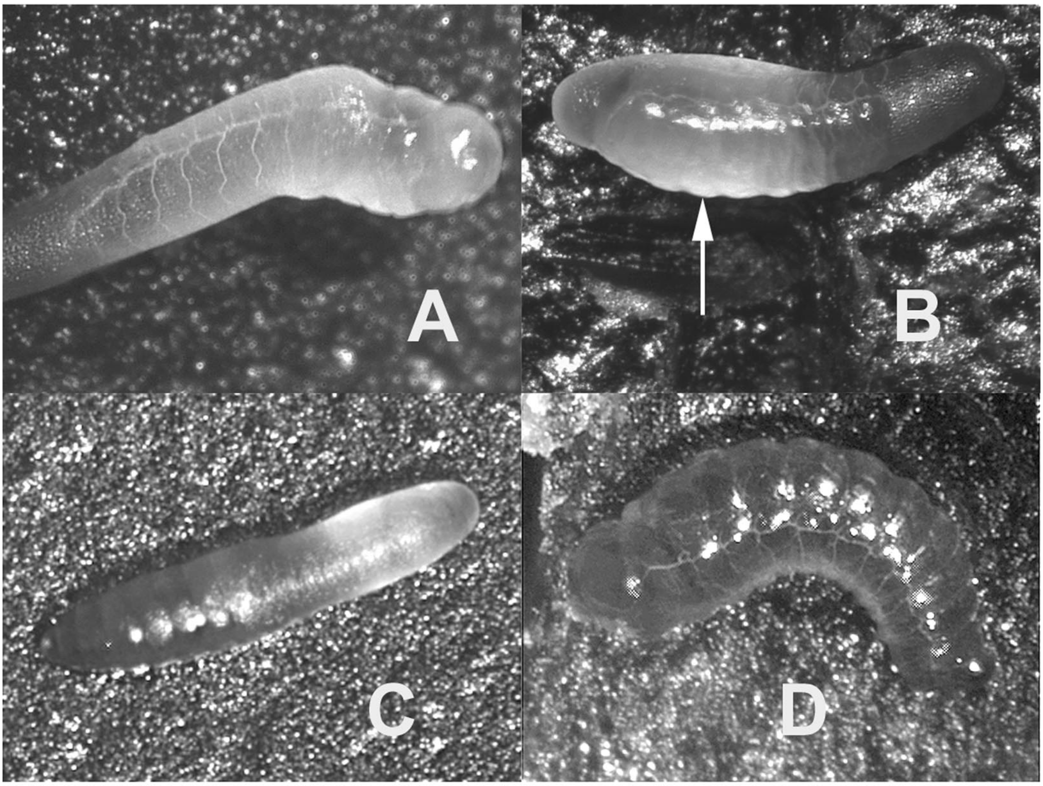

Fig. 4. Final stages of egg hatching. (A) Peristalsis of the abdomen, anterior to posterior—this may continue during the whole hatching process from appearance of the tracheal network to complete removal of the eggshell. (B and C) Embryos with partial digestion of the eggshell. (D) Completely hatched larva.

periods, 74 ± 34 min ($N = 7$), and may well force the digestion fluid onto the eggshell surface and spread it around.

Figure 4, B and C, shows an embryo partially through the hatching process. Embryo B has the more usual appearance, with the eggshell gone from the middle of the embryo and segments clearly visible (arrow). The head (left) and tail are still covered with vitelline membrane and chorion. Embryo C has started the digestion at one end and is about one-half released. Both of these patterns of digestion of the eggshell resulted in hatched larvae.

A completely hatched larva is shown in Fig. 4D. Initially, a small puddle of liquid was observed around the larva, but this quickly disappeared. When a small droplet of water was placed over a newly hatched larva, a remnant of eggshell was sometimes seen (unpublished data). This suggests that the eggshell is eventually completely digested by the secreted fluid.

Breathing by the larvae was often visible as moving reflections at the spiracles. The larvae continued to breathe for some time (301 ± 135 min, $N = 227$), but if they were not fed, as was the case in this study, they eventually died and began to disintegrate. It will be necessary to feed royal jelly or some other nutrient to hatched larvae before this time, because the intention is to use these larva to rear new queens. The standard process for queen rearing (grafting) involves transferring a very young larva, <2 d old, from the comb cell to a beeswax cup. Rows of these cups are hung upside down in a strong queenless colony, and the worker bees feed the larvae large amounts of royal jelly, extend the cell downward from the cup, and seal it for pupation.

Variation in hatch time was exaggerated by the range of ages of a group of eggs but was still considerable (Fig. 5). The mean hatch times for eggs from the nine different queens are reported in Table 1. Queens from nucleus colonies 6 and 13 were both from breeder B but were significantly different in time of hatching from the rest of the queens ($F = 47.06$; df = 8; $P < 0.0001$). Eggs laid by the queen in colony 6 hatched much later than other eggs, at ≈3.5 d, and eggs of queen 13 hatched early (68 h). Queen 13 only laid eggs for one of the observation periods, but queen 6 was one of the most frequent layers, and the hatching was consistently late. DuPraw (1961) also mentions eggs that hatched at 84 h but provided no data.

Based on these observations, it will be necessary to begin checking incubating eggs for signs of hatching at least as early as 66 h after oviposition. The majority of eggs hatch between then and 78 h. Before using any

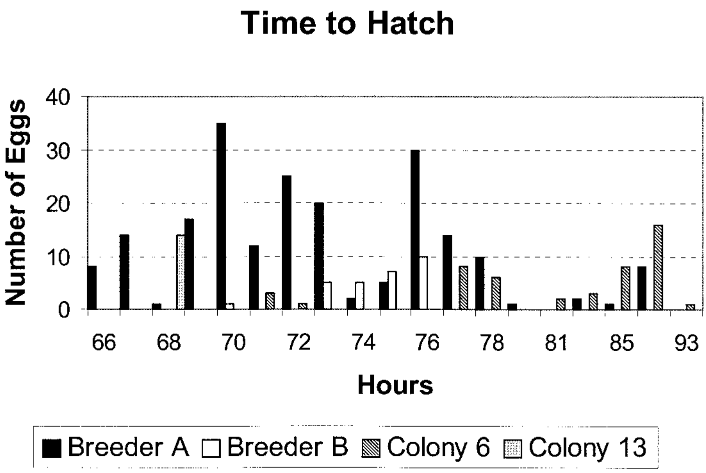

Fig. 5. The range of time to hatch for 550 eggs from two commercial breeder stocks, observed at 35°C and >95% RH. Colonies 6 and 13 (breeder B population) are shown separately, because their mean times were significantly different from all other colonies ($F = 47.06$; df = 8; $P < 0.001$).

queen for research on cryopreservation of embryos, it would be useful to determine the natural hatching time of her eggs so that observation times can be adjusted as needed when evaluating cryopreservation treatments. Once larvae have hatched, they will need to be fed within 2–3 h to keep them alive for 1–2 d, when they will be old enough to be used for queen rearing. Using a normal grafting tool to transfer these larvae to a queen cup for rearing by a colony should not be more difficult than transferring naturally reared larvae, but this will need to be demonstrated.

The mode of inheritance of the duration of the egg stage (time to hatching) would be useful information, because this measure could be a marker for selection of decreased development time. Decreased development time is proposed as an attribute of varroa mite resistant stocks, because fewer mites will be reared with bee pupae that hatch more quickly (Ifantitis 1983). Queens of the Africanized honey bee type emerge earlier than do queens from European stocks. Harbo et al. (1981) compared egg hatching times of Africanized and European bees and found that the Africanized eggs also hatched earlier. If a similar reduction in duration of life stage was applied to larvae and pupae as well, it could explain the observed differences in adult emergence between Africanized and European queens. Therefore, the time from oviposition to hatching would be an easily measured characteristic to select for an overall increased developmental rate.

Table 1. Mean and SD age of egg hatch for nine queens from two commercial breeders

Queen from colony no.	Breeder stock	Mean (h:m)	SD (h:m)
1	A	72:13	0:18
2	A	74:14	1:24
3	A	72:00	0:35
4	A	76:06	0:51
5	A	75:06	1:03
6	B	84:39 a	0:38
9	A	75:52	0:58
11	B	75:21	1:09
13	B	67:55 b	1:03

Values with letters are different from all others and from each other ($F = 47.06$; df = 8; $P < 0.001$).

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